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COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COC)

Scoping paper - The Tumour Microenvironment and its Role in Carcinogenicity

Background

1. The Committee has previously mentioned the importance of considering the influence of the immune system and pre-tumour cell microenvironment on the development of cancer. A short overview of the immunological and stromal cell modulations relevant to cancer risk discussed during the COC annual Horizon Scanning in November 2019 (CC/2019/13).

2. It was recognised that for such systems to be addressed, current testing strategies and approaches to risk assessment may need to be reconsidered. It was agreed that a position paper to explore available information to address these issues and where COC influence can best be targeted, was agreed as an initial way forward.

3. This scoping paper outlines aspects around the role of the tumour environment in carcinogenicity. Examples of potential effects of environmental chemicals are also given. The Committee is invited to consider what aspects to address and areas to work up for the proposed position paper.

Introduction

4. The history of cancer research from Boveri over 100 years ago (translated and annotated, Boveri, 2008) to more recent studies on the role of mutated oncogenes and tumour suppressor genes has shown that cancer development is driven by both genetic and epigenetic events. Recent research has further identified mutations in key genes in a range of cancers in different organs and tissues (Cieslik and Chinnalyan, 2020). Therefore, the assays developed for the risk assessment of potential carcinogens have been greatly influenced by these observations with the drivers being the ability to cause mutation and genotoxicity.

5. However, recent research has also suggested that although genetic, and epigenetic changes such as hypermethylation are of great importance, a number of non-genetic events are also involved in the aetiology of cancer (Smith et al, 2016). Hanahan et al. (2000; 2011) further identified and named them in their Hallmarks of Cancer as outlined below.

The Ten Hallmarks of Cancer

- *Genetic instability and mutation* – allowing changes in one cell to pass to daughter cells through mutation or epigenetic changes in the parent cell DNA.

- *Tumour-promoting inflammation* – helping cancer cells grow using the same growth signals which normal cells provide to each other during wound healing and embryonic growth; inflammation further contributes to the survival of malignant cells, angiogenesis, metastasis and the subversion of adaptive immunity.

- *Sustained proliferative signalling* – cancer cells appear to grow at an unlimited rate.

- *Insensitivity to anti-growth signals* – cancer cells are insensitive to anti-growth signals or withdrawal of normal growth signals.

- *Resistance to cell death* – cancer cells avoid the processes by which abnormal or redundant cells trigger apoptosis.

- *Replicative immortality* – cancer cells do not senesce or die after a limited number of cell divisions.

- *Dysregulated metabolism* – disrupting metabolism is needed to support the increased demands of rapid proliferation, thus enabling the development of cancer.

- *Angiogenesis* – eliciting new blood vessels to sustain growth.

- *Tissue invasion and metastasis* – invasive tumours creating a space to expand into normal tissue, while in situ or non-invasive cancers (e.g. breast ductal carcinoma in situ; carcinoma in situ in colon polyps) grow into pre-existing spaces.

- *Avoiding immune destruction* – tumour cells avoiding immune surveillance that would otherwise mark them out for destruction.

6. Some of these events, such as inflammation, angiogenesis and changes in immune response, identified in cancer development suggest that it is influenced by a number of different cell types besides the pre-tumour cells, and there has been an increasing level of research interest in the role the microenvironment plays in the mechanism by which tumour cells may be initiated and develop.

7. In the risk assessment of carcinogenic potential of chemicals, the traditional paradigm considering the reductionist, linear progress of chemical exposure to development of a tumour has led to problems when considering chemicals such as

non-genotoxins and endocrine disrupters, and potential low-dose, long-term exposure.

8. With recent advances in research, COC has now considered a more forward-looking approach which may be to move away from this simplistic classical model and consider and include the role of the tumour microenvironment in the process of carcinogenicity. This view is supported by the perception that there is now growing evidence that many of the effects of carcinogens occur through modulation of the tumour microenvironment. This had been comprehensively reviewed by Casey et al. (2015), which forms most of the basis of this scoping paper, and is attached at Annex A.

The tumour microenvironment

9. Early cancer development is associated with recruitment of a range of different cell types including multipotent stromal cells, epithelial cells, fibroblasts, vascular cells, inflammatory cells and a range of immune cells (including natural killer (NK) and T-cells) in an extracellular matrix. These different cell types produce and regulate a range of growth factors and signals, and other cellular modulators such as cytokines.

10. There is now considerable evidence that environmental carcinogens and other chemicals interact with the cells present in the tumour microenvironment. One of the main concepts is that, while the long-term effect of most carcinogens is increased cellular proliferation which may lead to cancer, they are also toxic to their target tissue (Casey et al., 2015). There is some evidence that this paradox may occur by the growth of pre-neoplastic lesions being promoted by a concomitant limitation of the proliferative capacity of the surrounding microenvironment.

11. Figure 1 of Annex A illustrates the complexity of the tumour microenvironment and summarises the range of cells and substances which may be present, and some of the active signals which they may produce.

Cell types and the tumour microenvironment

Blood vessels and vascular endothelium

12. The vasculature around the tumour cells is lined by a number of different cell types including fibroblasts, endothelial cells and pericytes. Endothelial cells are activated during tumour progression leading to angiogenesis which supplies increased oxygen and nutrients needed for tumour expansion. Angiogenesis is the expansion of the vascular network using endothelial cells. This is a different process from vasculogenesis whereby blood vessels are formed from progenitor cells in the embryo. There is specific gene expression in endothelial cells associated with angiogenesis which appear to be influenced by soluble factors released by macrophages and other immune cells in the microenvironment.

13. Vascular endothelial growth factor (VEGF) is a well-known mitogen which is critical for angiogenesis. A number of chemicals have been shown to affect the expression of VEGF, either in animal models of embryogenesis or lung tumours including nicotine, oestradiol and the known carcinogen, *N*-nitrosobis(2-hydroxypropyl)amine. Further studies are required to understand how chemicals, potentially long-term and low dose, regulate the expression of VEGF and other angiogenic regulators.

Extracellular Matrix (ECM) and stromal fibroblasts

14. Matrix metalloproteinases (MMPs) appear to be key to the production of the extracellular matrix (ECM) in the microenvironment and are often over-produced in tumour cells (and fibroblasts). Tumour cells undergo a developmental process called epithelial-mesenchymal transition whereby cells become invasive because they form cell-ECM rather than cell-cell interactions. Evidence suggests that MMPs are involved in this process. MMPs are regulated by cytokines (e.g. interleukin-1, IL-1), tumour necrosis factor-α (TNF-α), growth factors, bacterial components, hormones and mechanical stress. For example, some hormone receptor activation, e.g. aryl hydrocarbon receptor (AhR), oestrogen receptors (ER-α and ER-β), induces MMP production and it is possible that endocrine disrupting chemicals may act through these pathways.

15. Less is known about the stroma and the effect of changes such as tissue remodelling and chronic inflammation on the development of cancer. However, it does appear that changes in matrix composition and tissue architecture have a major role in cancer development. For example, people with liver cirrhosis are at increased risk of hepatocellular carcinoma and Casey et al. (2015) suggest that most lung cancers develop in people with pre-tumour disease, including chronic lung disorders such as emphysema and fibrosis.

16. A number of carcinogens are implicated in this tissue remodelling and inflammation which accompanies cancer development, particularly in lung cancer, including asbestos, tobacco smoke, some heavy metals, crystalline silica, some forms of radiation and certain organic chemicals. Little is known of any effects of combined low-dose exposure of multiple chemicals on this process. Chemicals may act to remodel tissues by stimulating the release of pro-fibrotic growth factors, cytokine and chemokines to change deposition of collagens and glycoproteins.

Cells of the Immune system and inflammation

17. The role of the immune system in cancer development is complex involving interactions between the innate immune system and adaptive cells, the production of soluble cytokines and signal factors, and other components of the tumour microenvironment (see Figure 2, Annex A). During cancer development, activation of adaptive immune cells may result in tumour cell death, while chronic activation of innate immune cells at sites of pre-neoplastic growth may enhance tumor development (de Visser and Coussens, 2006; Zhanget al., 2018).

18. The innate immune system can both promote and suppress tumourigenesis. Immune cells such as $CD8^+$ T-cells and natural killers (NK) have the ability to kill cancer cells and this may be a natural role for the system.

19. However, these immune responses have been shown to be suppressed by known carcinogens including polycyclic aromatic hydrocarbons such as dimethylbenz(a)anthracene (DMBA), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), one of the main carcinogens present in tobacco smoke. DMBA also suppresses both the cell-mediated and humoral immune response. A number of hormone-like chemicals have also been shown to modulate the immune response by mechanisms such as macrophage stimulation to produce cytokines. These chemicals include diethylstilboestrol, bis(2-ethylhexyl)phthalate and p-nonylphenol.

20. Chronic inflammation is associated with an increased risk of cancer formation in many tissues and it has been estimated that 20% of cancer deaths are caused by chronic inflammation, including bowel and lung cancer. The mechanisms by which inflammation promotes cancer are complex but probably involve a number of different cell-types in the tumour microenvironment. Cancer can be promoted by inflammation, via enhancing proliferative and survival signalling, the induction of invasion and metastasis.

21. The state of chronic inflammation attracts activated immune cells, including myeloid-derived suppressor cells and tumour-associated macrophages, that produce many cytokines such as interleukins (IL-6, IL-17), interferons (IFNs), Tumour necrosis factor (TNF) and transforming growth factor-beta (TGF-β) and bone morphogenic proteins (BMPs) which possess pro-tumorigenic properties. These proteins activate intracellular signalling pathways (such as NFκB and Wnt) in pre-neoplastic cells, which promote proliferation and inhibit apoptosis.

22. In addition, chronic inflammation also leads to oxidative stress resulting in the increased production of reactive oxygen species (ROS) that are associated with mutagenic effects. High levels of ROS can cause tissue damage and cell death while lower levels have been associated with increased proliferation. ROS is now thought to have a role as a second messenger and can stimulate the induction of VEGF and therefore affect angiogenesis, promote cellular proliferation, immune evasion and play a role in cell survival.

23. A number of different cell types in the tumour microenvironment especially cancer-associated fibroblasts and tumour infiltrating immune cells, are involved in the increased production of ROS. The role of ROS in the tumour microenvironment has recently been reviewed by Weinberg et al. (2019).

24. Overall, the role of the immune system in the microenvironment is complex with a balance between desirable anti-tumour responses and undesirable pro-tumour chronic inflammatory responses.

The Microbiome

25. The microbiota consists of the commensal bacteria and other microorganisms that colonise the epithelial surfaces of our body. These microorganisms have been shown to produce small molecules and metabolites that have both local and systemic effects on cancer development (Elinov et al., 2019). Recent research has revealed a complex interaction between the microbiota, the immune system and the tumour.

26. Some bacterial constituents of the gut microflora are involved in carcinogenesis. For example, *Helicobacter pylori* is an IARC Group 1 carcinogen (IARC 2012) which causes gastric cancer. Other examples of bacterial species with a potential association include *Fusobacterium spp* with colorectal adenocarcinoma, while patients with colon cancer also have increased number of *E. coli.*

27. It has been shown in mice that the human microbiota may interact with the immune system, for example, through induction of interferon-ϒ (IFN-ϒ)- secreting CD^{+}T-cells.

28. The components of the microbiota have also been shown to contribute to the responsiveness of chemotherapy treatment, by either increased efficacy or resistance (Elinov et al., 2019). This suggests that chemical carcinogens may also interact with the microbiota.

Present and future methodology

29. There are a number of *in vitro-in vivo* systems that have been devised for the study of the behaviour of the tumour microenvironment during the process of carcinogenesis. Many of these involve the transplantation of neo-plastic cells into different microenvironments.

30. These include transplantation of cells from different stages in the development of cancer into a normal tissue microenvironment or, one that is chemically growth restricted. For example, the liver of rats treated with retrorsine (a pyrrolizidine alkaloid which blocks the hepatocyte cell cycle) have been transplanted with pre-neoplastic hepatic cells derived from nodules. It is then possible to study tumour development in the presence of a restricted microenvironment.

31. Pre-clinical models of cancer include *in vivo* patient-derived xenografts where human tumours are implanted into mice. There are also *in vitro* models where 3D organ structures are constructed to simulate the pathology of tumours while also attempting to demonstrate the presence of some of the microenvironment. It may be possible for these organoids to be genetically manipulated by CRISPR-Cas technology to include the described mutated genes commonly present in different cancers. Different cell types such as immune, stroma and vascular endothelium can be integrated into these organoids to produce models of the tumour microenvironment.

32. It may also be possible to add some aspects of the microbiome to such models. These *in vitro* models can be used to study tumour development, including the behaviour of the cells of the microenvironment.

33. Studies on the microbiota have often involved use of gnotobiotic (germ-free) mice treated with microbiomes from humans, either healthy, or patients with a defined bacterial mixture. These rather 'low-tech' techniques have been used for many years. It is at present unclear whether these microbiota act exactly as those in a normal human environment and for the study of potential carcinogens, the chemicals would be handled by the mouse metabolism. The recent DNA technology can also be used to further "humanise "mice to produce better models.

34. Another challenge for research into the role of the microenvironment in tumour development is to integrate information from epidemiological population studies. Recent studies in molecular epidemiology have identified a number of biomarkers of genetic and epigenetic (such as methylation) events and exposure to genotoxic carcinogens (Chen et al., 2005: Vineis and Perera, 2007). Such information is important in the early detection of cancer and the identification of susceptible sub-groups. These studies could be expanded to investigate possible effects of the microenvironment: this could include biomarkers of immune response, inflammation, vascular signals and markers of histology.

Summary

35. Mutations in a number of key genes are of vital importance in the development of cancers in many tissues and organs. This has recently been clearly elucidated in a series of 22 papers by the Pan-Cancer Analysis of Whole Genomes (PCAWG) in the journal, Nature and other journals (for a commentary and references, see Cieslik and Chinnalyan, 2020). Therefore, mutational and genetic effects are vital in the assessment of the risks of potential carcinogens.

36. However, it has been shown that the development of cancer may also involve a number of stages which may not be driven by mutation alone, but, as outlined in here, may include inflammation, angiogenesis, changes in immune response and tissue invasion. The processes may involve different types of cells. These cells may be present in the microenvironment around the developing clonal cancer cells.

37. Chemicals may affect these cells and their interactions and subsequently affect the development of cancers. That being the case, COC is considering how to undertake a more holistic approach to the risk assessment of potential carcinogens including temporal and spatial aspects that take into account exposure to long-term low-doses of chemicals and also the effects of combined exposure to multiple chemicals.

38. The present regulatory methodology for the detection and risk assessment of potential carcinogens does not adequately consider all these complex mechanisms and interactions in the microenvironment, and new methodology will need to be

developed to consider the possible interactions of chemicals with these processes which may contribute to cancer.

Questions for the Committee

Members are asked to consider this paper and in particular:

i. Does the Committee wish to make any specific comments on aspects raised in this paper, or are there areas where further information should be sought?
ii. Are there any aspects Members are aware of that are not covered by the paper?
iii. How does the Committee wish to take forward a position paper on this area?

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Abbreviations

AhR - aryl hydrocarbon receptor

APC – antigen presenting cell

BMP - bone morphogenic proteins

DMBA - dimethylbenz(a)anthracene

ECM - extracellular matrix

IFN - interferon

MMP - Matrix metalloproteinase

NK - natural killer cells

NNK - 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

ROS - reactive oxygen species

TGF-β - transforming growth factor-beta

TNF - tumour necrosis factor

VEGF - vascular endothelial growth factor

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